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Differentiation and regeneration potential of mesenchymal progenitor cells derived from traumatized muscle tissue

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Abstract

Mesenchymal stem cell (MSC) therapy is a promising approach to promote tissue regeneration by either differentiating the MSCs into the desired cell type or by using their trophic functions to promote endogenous tissue repair. These strategies of regenerative medicine are limited by the availability of MSCs at the point of clinical care. Our laboratory has recently identified multipotent mesenchymal progenitor cells (MPCs) in traumatically injured muscle tissue, and the objective of this study was to compare these cells to a typical population of bone marrow derived MSCs. Our hypothesis was that the MPCs exhibit multilineage differentiation and expression of trophic properties that make functionally them equivalent to bone marrow derived MSCs for tissue regeneration therapies. Quantitative evaluation of their proliferation, metabolic activity, expression of characteristic cell-surface markers and baseline gene expression profile demonstrate substantial similarity between the two cell types. The MPCs were capable of differentiation into osteoblasts, adipocytes and chondrocytes, but they appeared to demonstrate limited lineage commitment compared to the bone marrow derived MSCs. The MPCs also exhibited trophic (*i.e.* immunoregulatory and pro-angiogenic) properties that were comparable to those of MSCs. These results suggest that the traumatized muscle derived MPCs may not be a direct substitute for bone marrow derived MSCs. However, because of their availability and abundance, particularly following orthopaedic injuries when traumatized muscle is available to harvest autologous cells, MPCs are a promising cell source for regenerative medicine therapies designed to take advantage of their trophic properties.

Keywords: mesenchymal stem cells • progenitor cells • tissue engineering • regenerative medicine • immunoregulation • angiogenesis

Introduction

Mesenchymal stem cells (MSCs) offer enormous promise in emerging strategies of cell-based therapeutics to promote the regeneration of damaged tissues and to modify the natural history of many diseases [1–4]. One attractive feature of MSCs is their ability to differentiate into multiple cell types, which makes them useful for tissue regeneration strategies to replace damaged, impaired or dead cells and tissues, either by cell replacement therapy [5–6] or in combination with a scaffold for tissue engineering [7]. MSCs also exert trophic functions in the environment of damaged tissues that promote endogenous wound healing mechanisms [8], such as promoting angiogenesis [9–10], reducing fibrosis [11] and modulating inflammation [12]. In particular, the immunoregulatory properties of MSCs have

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made them a useful therapeutic for the treatment of graft *versus* host disease [13]. Although the pro-regeneration functions of MSCs make them applicable for a variety of other clinical applications, there are significant challenges that limit their widespread use, including optimization of methods to harvest the cells, expand them *ex vivo*, and in the case of allogeneic MSCs, to transport and store the cells prior to implantation [14]. Many of these limitations could be overcome by using autogenic MSCs that can be harvested and used as a therapeutic agent at the point of clinical care [15].

Our laboratory has recently identified a novel population of cells in traumatically injured muscle tissue that resemble MSCs. Although multipotent stem cells have previously been isolated from untraumatized muscle using immuno-selective techniques [16], the plastic-adherent cells isolated from traumatized muscle can be harvested in substantially higher numbers [17]. We refer to this population as mesenchymal progenitor cells (MPCs) to indicate that these cells are likely to be the descendants of stem cells (*e.g.* pericytes [18]) but are proliferating in the tissue at the time of harvest. The morphology and cell surface epitope profiles of MPCs are similar to those of bone marrow derived MSCs, and they give rise to colony-forming unit fibroblasts, an indicator of a clonogenic, multipotent cell population [19]. Although the MPCs were capable of differentiation into osteoblasts, adipocytes and chondrocytes, they appeared to have a distinct osteogenic gene expression profile, which likely reflects their different tissue of origin and *in vivo* function [20]. It has been suggested that non-muscle progenitors participate in the process of muscle regeneration by facilitating the reparative function of myoblasts and myofibroblasts [21] and that the migration of MPCs into the traumatized muscle may be a part of the normal wound healing response [22].

The traumatized muscle derived MPCs could be a useful cell source for cell-based therapies that follow musculoskeletal injury, particularly those related to orthopaedic reconstruction [23]. MPCs have been harvested from surgically debrided tissues from the wound margins of extremity injuries [19], which would provide an autogenic cell source that does not require a separate procedure for cell collection. Because the MPCs are harvested at relatively high concentrations, they may not require *ex vivo* expansion and could be ready for use almost immediately. However, there have been no quantitative comparisons of the MPCs to bone marrow derived MSCs to evaluate whether the MPCs are a suitable substitute cell type. Furthermore, the ability of MPCs to exhibit trophic properties has not yet been investigated. The overall hypothesis of this study was that traumatized muscle derived MPCs exhibit multilineage differentiation and expression of trophic properties that make them equivalent to bone marrow derived MSCs to enhance tissue regeneration. Our specific aims were: (1) to compare the phenotype characteristics of MPCs to bone marrow derived MSCs, (2) to quantify the ability of the MPCs to differentiate into osteoblasts, adipocytes and chondrocytes and (3) to determine whether MPCs exhibit trophic properties that are characteristic of MSCs.

Materials and methods

MPC harvest

Traumatically injured muscle was collected with informed consent and Institutional Review Board approval from the Walter Reed Army Medical Center using a previously described method [17, 19]. Patients were considered for inclusion in this study if they had sustained traumatic injury with extensive soft tissue extremity wounds ($n = 24$; age: 24.4 $\pm$ 4.7; sex: 100% male). Traumatized muscle was obtained from the healthy wound margin during serial debridement following orthopaedic trauma. Serial sections of the traumatized muscle were stained with haematoxylin and eosin to evaluate the tissue morphology and compared to control muscle sections (Biogenex, San Ramon, CA, USA). Approximately 200 mg of the tissue was minced, incubated at 37°C for 2 hrs with gentle agitation in digestion medium (DMEM with 0.5 mg/ml collagenase 2, Worthington Biosciences, Lakewood, NJ, USA) and passed through a 40 μm cell strainer. The cells were pelleted by centrifugation, resuspended and plated in a T150 tissue culture flask in DMEM supplemented with 10% foetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) and 5 units/ml of penicillin, streptomycin and fungizone (PSF, Invitrogen). After 2 hrs, the non-adherent cells were removed by washing with phosphate-buffered saline (PBS, Invitrogen). The adherent cells were cultured in growth medium (DMEM supplemented with 10% FBS and 1 unit/ml of PSF) and washed daily with PBS until subculture when tightly packed colony forming units were observed. Subsequent subcultures were performed when the cells were approximately 85% confluent. Bone marrow derived MSCs were harvested from femoral heads obtained from total hip arthroplasties using an established technique [24] and with the consent of the patients ($n = 14$; age: 54.9 $\pm$ 8.7; sex: 42.8% male) following an Institutional Review Board approved protocol at the University of Washington. All cells were cryopreserved at the end of the first passage, and multiple biological replicates of each experiment were performed by thawing the cells simultaneously. Unless otherwise noted, all experiments were performed with third or fourth passage cultures.

Proliferation and metabolic activity assays

Cells were plated in 24-well plates at an initial density of 1000 cells/cm^2. Cell number at the prescribed time-points was determined on the basis of dsDNA concentration using the PicoGreen assay (Invitrogen), and the doubling time was estimated using an exponential fit. Cellular metabolism based on the output of mitochondrial reductase was estimated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, Saint Louis, MO, USA). At the prescribed time-points, MTT was added to the medium in 4 replicate wells to a final concentration of 1 mg/ml, and the cells were incubated at 37°C for 2 hrs. The medium was aspirated, and the incorporated MTT reduction product was eluted with dimethyl sulfoxide (DMSO) and quantified spectrophotometrically (A_{550}) using a microtiter plate reader (Synergy HT, Bio-Tek, Winooski, VT, USA).

Immunophenotyping

Flow cytometry was performed on cells at the end of the second passage as previously described [17]. Briefly, 100,000 cells were incubated with approximately 0.4 μg of phycoerythrin (PE) conjugated antibodies in

1.0 ml of flow cytometry buffer (FCM) buffer [0.1% bovine serum albumin and 0.01% sodium azide in Hank's buffered saline solution (HBSS)]. All antibodies were mouse IgG1, κ-isotype and reactive against human antigens (isotype control: clone MOPC-2I; CD14: clone M5A2; CD19: Clone HIB19; CD34: clone 563; CD45: clone HI30; CD49d: clone 9F10; CD73: clone AD2; CD79a: clone HM47; CD90: clone 5E10; CD146: clone P1H12; HLA-DR: clone TU36 – BD Biosciences, San Jose, CA, USA; CD105: clone 6N6 – Serotec, Oxford, UK). The cells were incubated in the dark at 4°C for 40 min., washed once and resuspended in 100 μl of FCM buffer, and the fluorescence intensity profiles of the cells were analysed using a FACSCalibur flow cytometer (BD Biosciences).

Differentiation assays

The MPCs and MSCs were plated onto tissue culture plastic and cultured for up to 28 days in either (1) osteogenic induction medium (OM) containing growth medium (GM) supplemented with 10 mM β-glycerophosphate (Sigma), 50 μg/ml ascorbic acid (Sigma), 10 nM 1,25-dihydroxyvitamin D_3 (Biomol, International, Plymouth Meeting, PA, USA) and 0.01 μM dexamethasone (dex, Sigma) or (2) adipogenic induction medium (AM) containing GM supplemented with 0.5 mM IBMX (Arcos Organics, Geel, Belguim), 1 μM dex and 1 μg/ml insulin (Sigma). Chondrogenic differentiation was performed in pellet cultures containing 2.5×10^5 cells per pellet and by culturing the cells in chondrogenic medium (CM) containing DMEM supplemented with 1% Insulin-Transferrin-Selenium Premix (ITS Premix) (BD Biosciences), 40 μg/ml L-proline, 10 ng/ml transforming growth factor-β_3 (TGF-β_3, R&D Systems, Minneapolis, MN, USA), and 0.1 μM dex for 21 days. Osteogenesis was assessed using a bb Fast Blue alkaline phosphatase (ALP) kit (Sigma) and by staining mineralized matrix with alizarin red. ALP activity was quantified with an ELISA-based assay [20]. Adipogenesis was assessed using oil red O to stain for intracellular lipid accumulation, which was eluted with isopropanol for spectrophotometric quantification (A_{510}). Chondrogenesis was assayed histochemically by staining for sulphated glycosaminoglycans (GAGs) with alcian blue, and immunohistochemically by staining for collagen type II and aggrecan. GAG production was quantified using the Blyscan assay kit (Biocolor, Carrickfergus, UK).

qRT-PCR array analysis

Cells were lysed in TRIzol (Invitrogen), homogenized using QiaShredder columns (Qiagen, Hilden, Germany) and total RNA was extracted according to the manufacturer's protocol, purified using RNeasy Mini columns (Qiagen), and RNA concentrations were estimated on the basis of A_{260}. Real-time RT-PCR analysis was performed with SYBR Green (Bio-Rad, Hercules, CA, USA) and a BioRad iCycler iQ real-time PCR detection system and gene expression was normalized to GAPDH using RT^2 PCR arrays for MSC biology (SABiosciences, Frederick, MD, USA).

Mixed lymphocyte reaction

Peripheral blood from healthy human donors was collected into heparinized containers (BD Biosciences), and mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density-gradient centrifugation as described previously [25]. Responder human PBMC were resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% FBS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) and 50 μM 2-mercaptoethanol (Invitrogen). PBMCs were seeded in triplicates at 1×10^5 cells/100 μl/well in 96-well round bottom plates (BD Biosciences). Phytohemagglutinin was used at 5 μg/ml as a positive control mitogen to induce T-cell proliferation. MPCs were added at prescribed concentrations to obtain a 300 μl final volume. After 3 days of incubation, 1 μCi/well [^{3}H]-thymidine (GE Healthcare, Piscataway, NJ, USA) was added overnight and radioactivity incorporation was determined by liquid scintillation counting.

Vascular stability assays

MPCs were cultured for 3 days in expansion medium and then with serum-free medium. After 1, 2 or 4 days, the media were then collected and concentrated by centrifugation in spin columns (Amicon Ultra-15 3 kD NMWL; typical concentration yields: 20–40× by volume) to generate conditioned media (CondM). Aliquots of the CondM were analysed by SDS-PAGE and Western blotting for vascular endothelial growth factor (VEGF). Human microvascular endothelial cells (HMEC)-1 (Center for Disease Control [26, 27]) were cultured in CondM that had been reconstituted to 1× in serum free medium supplemented with EGM-2-MV bullet kits (Cambrex, East Rutherford, NJ, USA) [28]. After 48 or 72 hrs, cell proliferation was quantified using the MTS Cell Proliferation Assay (Promega, Madison, WI, USA).

Statistical methods

Statistical significance for all tests was assigned to $P < 0.05$ except for RT-PCR array gene expression levels, for which statistical significance was assigned to $P < 0.018$ to limit the false discovery rate.

Results

The phenotype of traumatized muscle derived MPCs was similar to other populations of MSCs

The traumatized muscle exhibited substantial histological evidence of tissue damage (Fig. 1A). Within 2 hrs of plating the cells harvested from this tissue, adherent MPCs with an MSC-like morphology were observed for all of the 26 traumatized muscle tissue samples included in this study (Fig. 1B). The MPCs were present at substantially greater numbers in the traumatized muscle than MSCs are present in the bone marrow, and the traumatized muscle also yielded a greater number of MPCs that formed ALP^+ colonies (Table 1). There was no significant difference in the proliferation rate of the MPCs compared to bone marrow derived MSC (Fig. 1C), although the metabolic activity of the MPCs was significantly higher (Fig. 1D). The cell surface epitope profile of the MPCs met the minimum criteria established for MSC populations [29] (Fig. 2A), and there was no significant difference in the fluorescence intensities of the selected positive markers. Although the intensity of CD49d staining was low on the MPCs, greater than 97% of the MPCs expressed this surface marker, which is not uniformly expressed on bone marrow derived MSCs [30]. Fewer than 5% of cells from either population expressed the pericyte marker CD146.

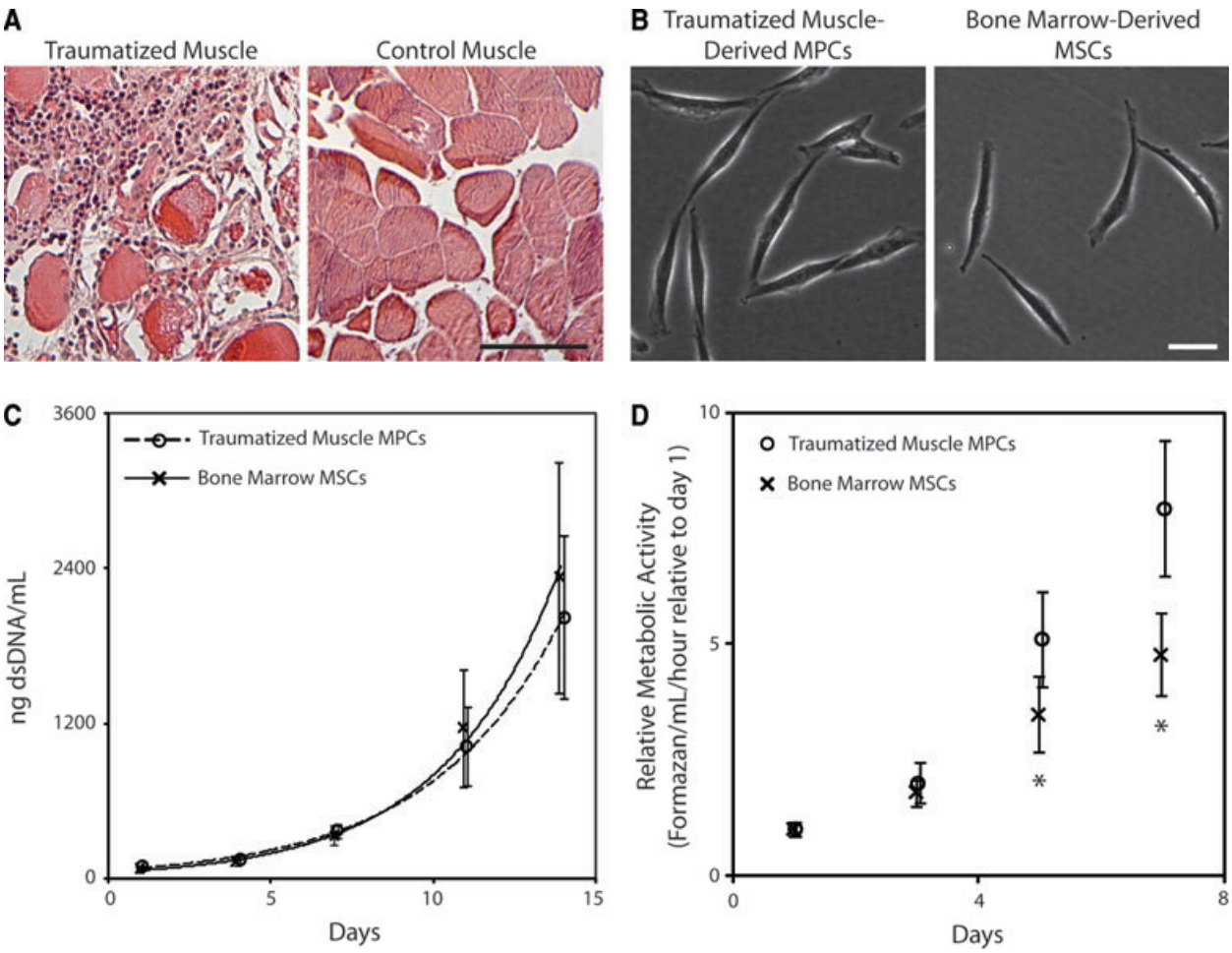


Fig. 1 Morphology, proliferation and metabolism of traumatized muscle derived MPCs. (**A**) Representative sections of the traumatized muscle demonstrating the extent of tissue damage compared to control muscle. Bar = 200 μm; haematoxylin and eosin. (**B**) MPC morphology was similar to that of bone marrow derived MSCs under phase contrast microscopy. Bar = 50 μm. (**C**) The proliferation of MPCs and MSCs was assayed on the basis of dsDNA, and no significant differences were detected. (**D**) Metabolic activity was measured on the basis of the chemical reduction capacity, and 5 days after seeding, a significant difference was detected between MPCs and MSCs. $^{*}P < 0.05$, Student's t-tests with $n = 4$.

Multilineage differentiation activity of MPCs was lower than bone marrow MSCs

Under appropriate induction conditions, both cell types exhibited evidence of osteogenic, adipogenic and chondrogenic differentiation (Fig. 3A). The MPCs up-regulated their surface expression of ALP and began to generate a mineralized matrix under osteogenic conditions (visualized with alizarin red), accumulated intracellular lipid stores under adipogenic conditions (visualized with oil red O), and they began to express and produce collagen type II and sulphated GAGs (visualized with alcian blue), including aggrecan, under chondrogenic conditions.

Upon quantification, the MPCs appeared to exhibit a lower level of differentiation than bone marrow derived MSCs. Although, the MPCs accumulated more intracellular lipids than MSCs under adipogenic conditions (Fig. 3C), the up-regulation of surface ALP in MPCs was significantly lower than MSCs under osteogenic conditions (Fig. 3B). Chondrogenic differentiation by MPCs was particularly limited, as they produced significantly smaller pellets, less sGAG per cell, and less overall sGAG than the bone marrow derived MSCs (Fig. 4D–F).

MPCs exhibit regenerative properties that are characteristic of MSCs

The gene expression profile of traumatized muscle derived MPCs was nearly identical to that of bone marrow derived MSCs

Table 1 Properties of traumatized muscle derived MPCs and bone marrow derived MSCs

	Traumatized muscle derived MPCs	Bone marrow derived MSCs
Adherent cells/million nucleated cells	370,000	5–50†
Adherent cells/g of tissue	1,980,000	350–1600‡
ALP^+ colonies/g of tissue	4300‡	4250†
dsDNA doubling time (days)	2.81	2.45
Metabolic activity doubling time (days)	3.56*	2.55*

†Values reported in [31, 32].
‡Value reported in [20].
*$P < 0.01$, Student's t-test with $n = 4$.

(Fig. 4A). No difference was detected between the two cell types in gene-expression levels of the MSC markers *CD44* and *ENG* (CD105; Fig. 4C), or baseline expression of *PPARG2*, *SOX9* and *RUNX2*, the master regulators of adipogenesis, chondrogenesis and osteogenesis, respectively (Fig. 4D). Notable differences in the gene expression profiles include *THY1* (CD90) and *NES* (nestin), which are expressed at significantly higher levels in MPCs, and *VCAM1* (vascular cellular adhesion molecule-1), which is expressed at significantly higher levels in bone marrow derived MSCs (Fig. 4B).

The MPCs also expressed similar levels of genes associated with the regenerative functions of MSCs. There was no significant difference in the gene expression of the immunoregulatory genes *IL6*, *IL10*, *HGF* (hepatocyte growth factor) or *TGFB3* between the MPCs and MSCs, while *IFNG* (interferon-γ) was expressed at slightly but significantly higher level in MPCs (Fig. 5A). The immunoregulatory function of the MPCs was verified using a mixed lymphocyte reaction assay (Fig. 5B). The MPCs attenuated T-cell proliferation in response to antigen stimulation in a dose dependent manner, although MSCs appeared to generate greater attenuation of the immune response. The MPCs and MSCs expressed nearly identical levels of *FGF2* (fibroblast growth factor-2), *EGF* (epithelial growth factor) and *VEGFA*, genes associated with vascular maintenance (Fig. 6A), and the production of VEGFA, a potent angiogenic factor, by MPCs was verified in Western blots (Fig. 6B). The pro-angiogenic potential of MPCs was assayed by measuring the effect of their secreted factors on the proliferation of microvascular endothelial cells (Fig. 6C). Similar to bone marrow derived MSCs, the MPCs secreted factors that significantly increased microvascular endothelial cell proliferation within 48 hrs of initial seeding, and there were no significant differences between the effects of the MPC or MSC secreted factors.

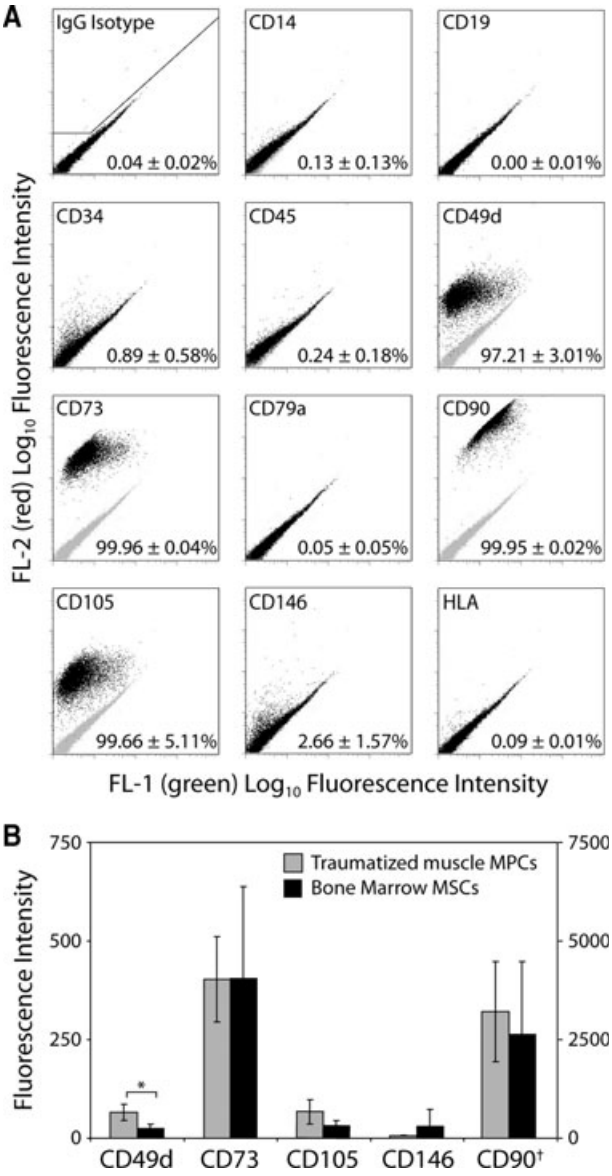


Fig. 2 Cell surface epitope profile of traumatized muscle derived MPCs. (**A**) MPC cell surface marker expression (black dots) compared to isotype control (grey dots) were compared using flow cytometry. All antibodies were PE conjugated (red), and the percentage of events with elevated FL-2 fluorescence is indicated in each panel (mean ± S.D. for $n = 4$). (**B**) The fluorescence intensity of positive surface markers was compared between MPCs and bone marrow derived MSCs. *$P < 0.05$, Student's *t*-tests with $n = 4$, †the fluorescence intensity of CD90 is 10× greater than the other surface markers and is depicted using the right axis.

Discussion

MSCs may be a useful cell type for a variety of applications in regenerative medicine, although there is currently no clear

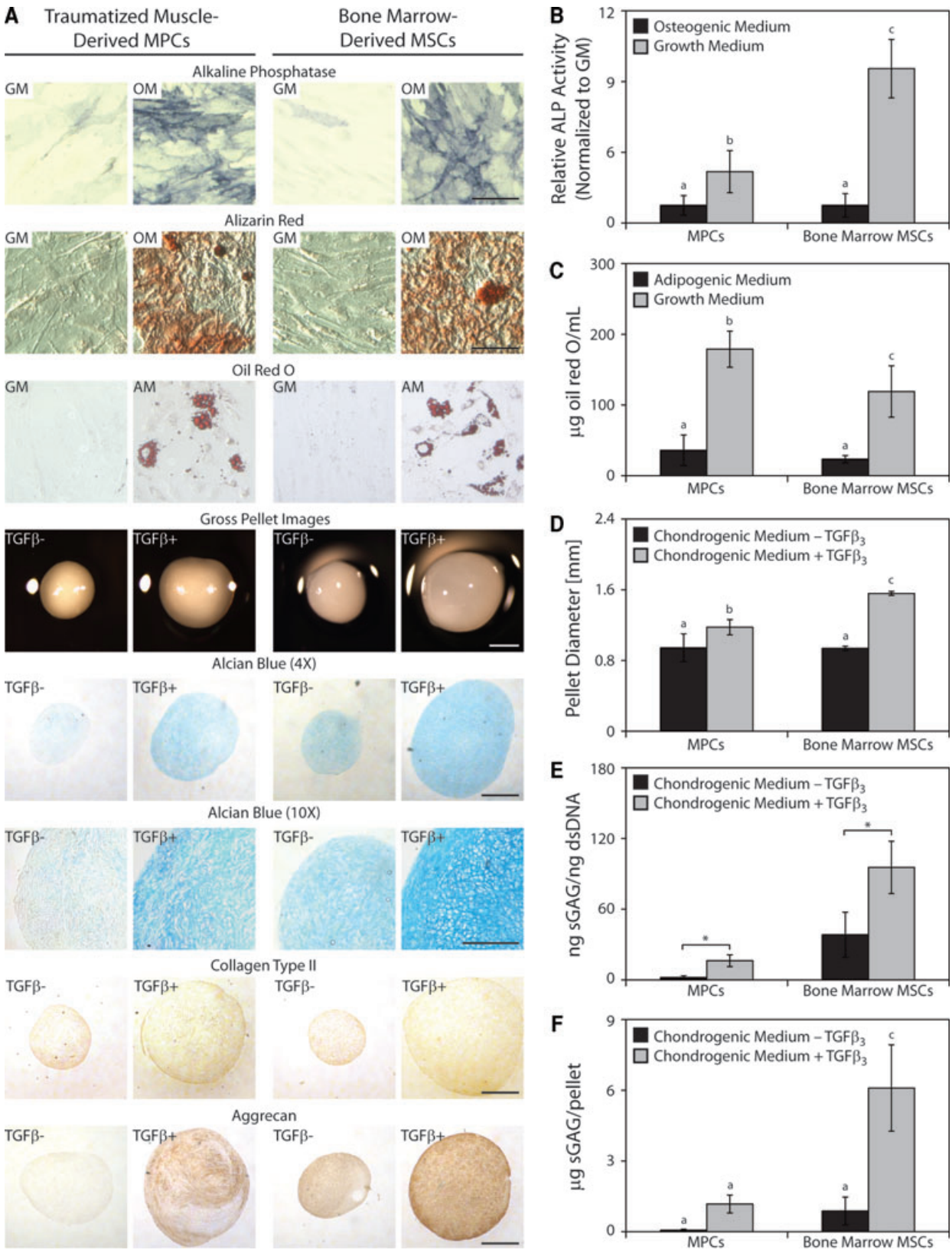
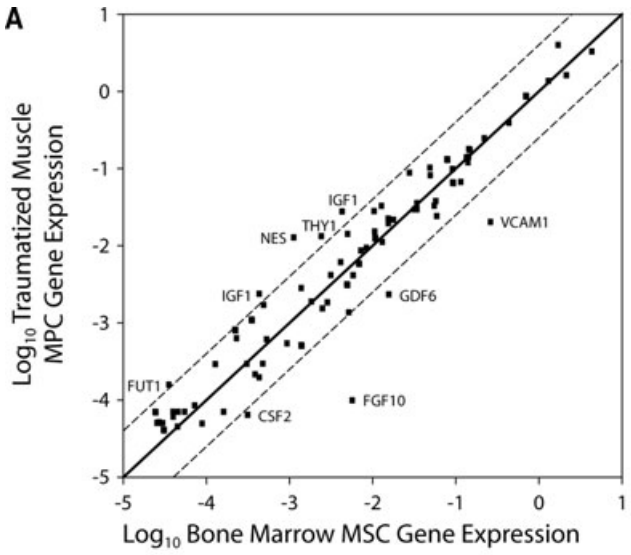


Fig. 3 Trilineage differentiation of traumatized muscle derived MPCs. (**A**) Histological evidence of differentiation in MPCs compared to bone marrow derived MSCs grown in GM, OM, AM and chondrogenic induction medium with (TGF-β^{+}) and without TGF-β_3 (TGF-β^{-}). Scale bars: ALP, alizarin red and oil red O: 100 μm. Gross pellet: 500 μm. Whole pellet section: 400 μm. 10× pellet: 200 μm. (**B**–**F**) Quantitative assays of differentiation: (**B**) ALP activity assay for osteogenic differentiation, (**C**) oil red O inclusion assay for adipogenesis, (**D**) pellet size, (**E**) total sGAG production and (**F**) normalized sGAG production. a, b and c: $P < 0.05$ with one-way ANOVA and Student-Newman-Keuls (SNK) multiple comparison tests and $n = 4$, *$P < 0.05$, Student's t-tests with $n = 4$.



	Fold Difference	p-value
FGF10	-59.0	0.03
VCAM1	-13.3	0.02
GDF6	-6.9	0.03
CSF2	-5.0	0.40
EGF	-2.3	0.05
IFNG	1.5	0.01
BMP7	2.8	0.03
BMP6	3.1	0.02
BDNF	3.6	0.01
THY1	5.3	0.04
IGF1	5.4	0.18
TGFB3	6.3	0.29
NES	11.0	0.05

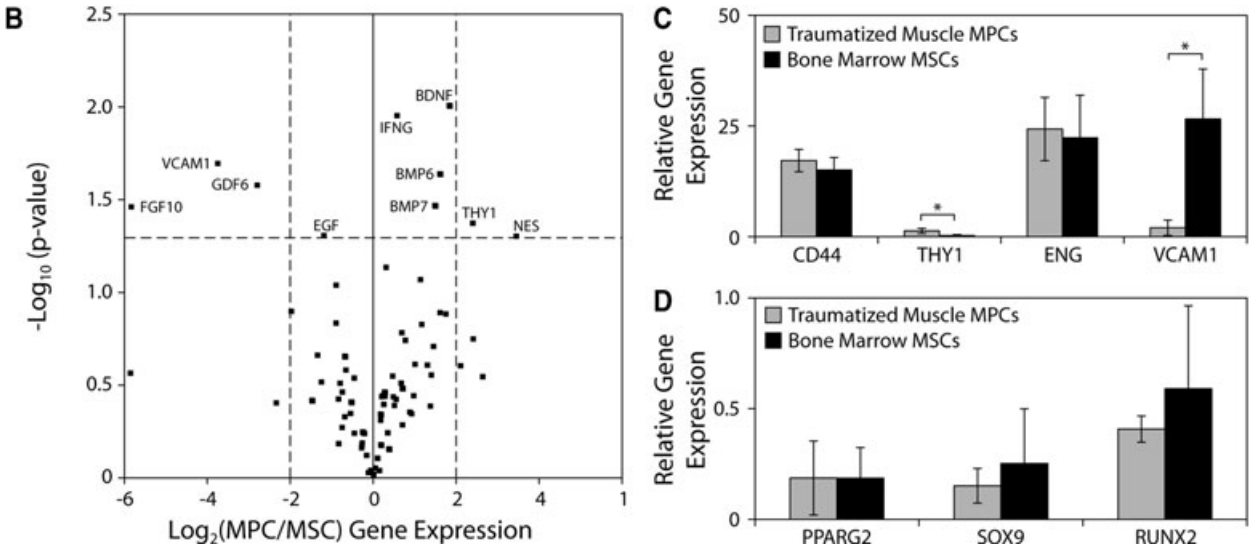


Fig. 4 qPCR array for MSC genes. (**A**) Comparison of the gene expression profiles for traumatized muscle derived MPCs and bone marrow derived MSCs. Genes with 4-fold differential expression (indicated by dashed lines) are labelled and select genes are listed in the adjacent table. (**B**) A volcano plot comparing the fold-difference in cytokine gene expression (*x*-axis; vertical dashed lines indicating 4-fold differential expression) to the statistical significance (*y*-axis; horizontal dashed line indicating $P < 0.05$). Bar graphs comparing: (**C**) specific MSC Markers (*THY1* = CD90, *ENG* = CD105 and *VCAM1* = CD106) and (**D**) baseline expression of master regulators associated with trilineage differentiation potential. *$P < 0.05$, Student's t-tests with $n = 3$.

consensus on the best method of sourcing and isolating MSCs for clinical use. In this study, we have evaluated a recently identified population of traumatized muscle derived MPCs by quantitatively comparing them to a typical population of bone marrow derived MSCs. The traumatized muscle tissue is an attractive source of autolgous cells with MSC-like properties, as it is readily obtained

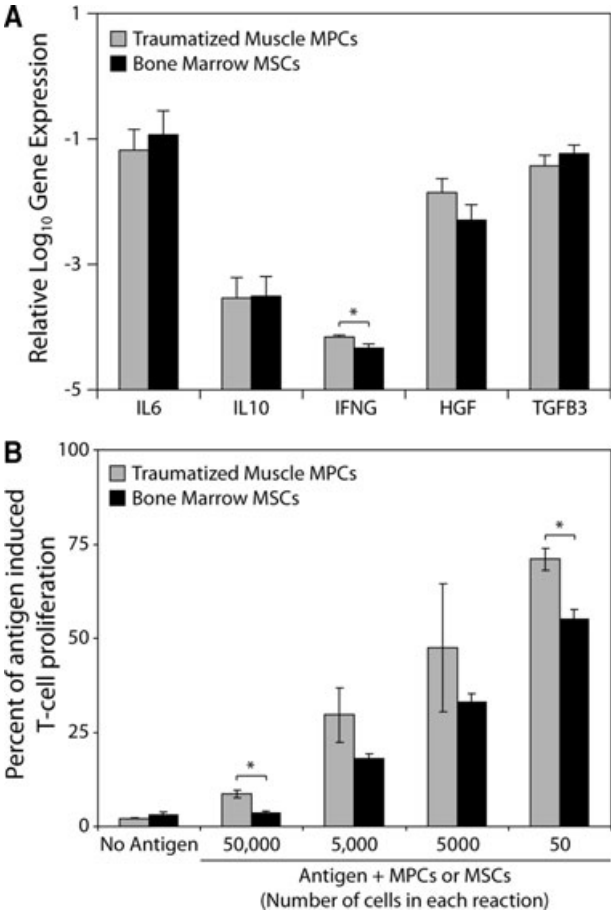


Fig. 5 Immunosuppression by traumatized muscle derived MPCs. (**A**) Comparison of immunoregulatory gene expression between MPCs and bone marrow derived MSCs. (**B**) T-cell proliferation in a mixed lymphocyte reaction plotted as a percentage of total proliferation in response to an antigen without modulation by MPCs or MSCs. $^*P < 0.05$, Student's t-tests and $n = 3$.

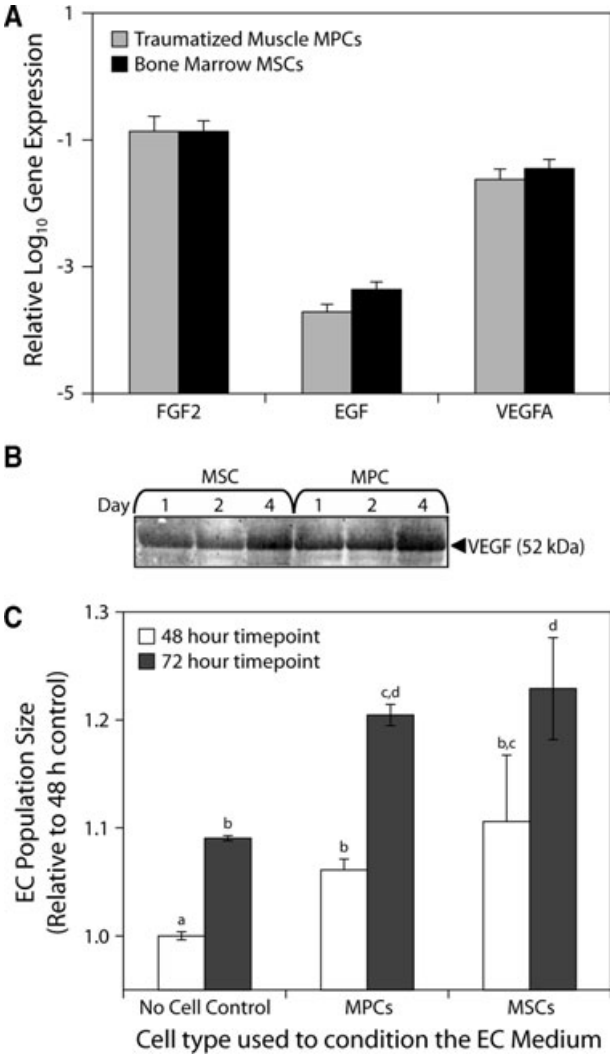


Fig. 6 Vascular maintenance associated with traumatized muscle derived MPCs. (**A**) Comparison of angiogenic gene expression between MPCs and bone marrow derived MSCs. (**B**) Western blots to verify VEGF protein expression in the cell supernatants at days 1, 2 and 4. (**C**) Proliferation of microvascular endothelial cells at 48 and 72 hr time-points. The cells were cultured in media that had been conditioned by MPCs, MSCs or no cells for 4 days prior to plating. a, b, c and d: $P < 0.05$, one-way ANOVA and SNK multiple comparisons and $n = 4$.

in a clinical setting following orthopaedic injury without requiring additional tissue harvesting procedures [33]. We have demonstrated that MPCs can be harvested at high concentrations from traumatized muscle, and the proliferation rate, cell surface epitope profile and gene expression profile of the MPCs is substantially similar to that of bone marrow derived MSCs. The MPCs also appear to maintain a higher level of metabolic activity relative to the MSCs. While they differentiate easily into adipocytes, the MPCs exhibit more limited differentiation into osteoblasts and chondrocytes. Finally, the MPCs express factors that modulate inflammatory responses and promote angiogenesis at functional levels that are comparable to the MSCs. Taken together, these results suggest that while traumatized muscle derived MPCs may not be a direct substitute for bone marrow derived MSCs, they perform specific trophic functions that will make them useful in regenerative medicine applications.

Several aspects of this study support our conclusions. First, both histological and quantitative analyses of the differentiation assays were included in our investigation. Compared to our previous qRT-PCR study showing the up-regulation of lineage specific genes by MPCs under the appropriate conditions [17, 19], the protein-level assays reported in this study provide a more precise measure of lineage adoption. Using these higher sensitivity assays, we were able to observe the limitations of the MPCs to differentiate into osteoblasts and chondrocytes relative to the MSCs.

Second, in addition to performing the *in vitro* differentiation assays on this novel population of MSC-like cells, we also investigated the trophic function of these cells, which are assumed to constitute at least part of the therapeutic benefit provided by MSCs. In addition to looking at the expression of genes associated with these functions, we performed functional assays to verify the activity of these trophic mechanisms by the traumatized muscle derived MPCs. Finally, all of the experiments in this study were performed with human cells from a clinically relevant source. As a result, our findings are directly applicable to the development of therapies that can take advantage of this cell type.

Despite these strengths, a few caveats should be noted. First, this study was designed to investigate the spectrum of regenerative functions that MPCs are capable of performing, but it did not generate any insight into the mechanisms by which the MPCs were able perform these functions. Many of these mechanisms have been identified in MSCs, and it is tempting to assume that they are conserved between the two cell types. We are currently performing more comprehensive studies that will investigate the mechanism of these functions in greater detail using additional experimental outputs. However, the present study was useful in that it provides a broad and quantitative comparison between the MPCs and bone marrow derived MSCs. Second, all of the experiments in this study were performed *in vitro*, and further investigation will be necessary to verify that the traumatized muscle derived MPCs will exhibit the same regenerative functions *in vivo*. Finally, the MPCs and MSCs were not age and sex matched due to limitations imposed by the demographics of the patients providing our tissue samples, in view of previous reports on the effects of age and sex on stem and progenitor cell function, the differences between the MPCs and MSCs reported here should be evaluated with caution.

Variations in differentiation potential [34] and trophic function [35, 36] of MSCs have previously been correlated with the age and sex of the donors from which the cells were harvested. The precise mechanism leading to these changes in MSC function are not completely understood, although age related changes have been attributed to telomere shortening [37], and the sex dimorphisms appear to be generated by preconditioning of the MSCs by sex hormones prior to harvest [38]. In the context of these donor effects, the differences in traumatized muscle derived MPCs and bone marrow derived MSCs can be examined in greater detail. The substantially higher yield of harvested MPCs relative to MSCs is not consistent with the changes in cellularity that might be attributable to donor age or sex [38, 39], suggesting that the difference in cellularity is dominated by the tissue of origin and the cellular responses to injury. However, despite the donor mismatch, no significant differences in the proliferation rate or expression of lineage specific genes were observed between the two cell types. These results suggest that the difference in mean age between the two donor groups had a small effect on these outputs relative to the overall biological variability. We did find evidence of a potential sexual dimorphism in that the MSCs, which contained cells from female donors, appeared to more effectively suppress T-cell proliferation. However, the difference in immunosuppression between the cell types was slight, and only significantly different at two cell concentrations. The angiogenic function of the MPCs and MSCs also appeared to be equivalent. Taken together, the effects of donor sex also appear to be small relative to the biological variability. Finally, the impaired osteogenic and chondrogenic differentiation of the MPCs relative to MSCs might be due to the presence of females in the MSC group, but this result is inconsistent with the expected age-related effects [40]. As a result, these differences in the differentiation potential might be dominated by the difference in cell type.

Other progenitor cells with properties similar to MSCs have been harvested from human skeletal muscle tissue. Using immunoselection techniques, a population of cells can be isolated from digested muscle tissue that can differentiate into osteoblasts, adipocytes and chondrocytes, as well as into myoblasts [41]. Termed myoendothelial cells due to the myogenic (CD56) and endothelial cell (CD34 and CD144) markers that distinguish this cell type, they also promote regeneration of skeletal [16] and cardiac [42] muscle tissue, in part by secreting pro-angiogenic and pro-survival factors that promote the endogenous repair mechanisms. These cells demonstrate many characteristics of the muscle-derived stem cells (MDSCs), which can be isolated from murine skeletal muscle [43], although a direct equivalent of MDCSs has not been identified in human muscle tissue. It is noteworthy that the MDSC cell type is isolated on the basis of its slow adherence to tissue culture plastic during the harvesting procedure [44], in contrast to the traumatized muscle derived MPCs, which rapidly adhere to the tissue culture plastic in less than 2 hrs.

In human beings, it is assumed that the myoendothelial cells are related to pericytes, as both cell types share the perivascular niche. The pericytes also closely resemble MSCs *in vitro*, and there has been recent, compelling evidence that pericytes can be harvested from various tissues throughout the body and induced to exhibit MSC characteristics [18]. The MPCs may be an activated descendent of the myoendothelial/pericyte cell types that have (1) been activated in response to traumatic injury, (2) down-regulated the expression of surface proteins required for their vascular niche [45] and (3) began to proliferate in the tissue [22]. There is also recent evidence indicating that a multipotential progenitor cell population may be derived from the vasculature *via* epithelial to mesenchymal transition in response to injury [46]. Taken together, these studies suggest that the MPCs may arise from the vasculature in large numbers following trauma, and they may participate in the wound healing process by secreting trophic factors to promote tissue regeneration by mechanisms similar to those used by the myoendothelial cells and pericytes [47].

The MPCs may also be descendants of bone marrow derived MSCs that entered the traumatized tissue *via* the bloodstream while homing to the site of injury [48]. Some differences were detected between the MPCs and bone marrow derived MSCs, and these differences may reflect the tissues from which they were harvested and the extracellular environment immediately prior to harvest. The MPCs exhibited higher metabolic activity than the MSCs, which may indicate that they undergo mitochondrial biogenesis in response to injury and to prepare them for their

contribution to the wound healing response [49]. The differences in the baseline gene expression profile may also be justified given that MPCs were in the regenerating muscle tissue at the time of harvest, whereas the MSCs were in their bone marrow niche [20]. For example, the MSCs expressed higher levels of *VCAM1*, which is characteristic of genes associated with bone physiology and maintenance by the marrow stroma [50], whereas the MPCs expressed higher levels of genes that indicate neuromuscular differentiation: *THY1* [51] and *NES* [52]. It was not possible to definitively trace the origin of the MPCs to the muscle tissue or the bone marrow without using an *in vivo* injury model. However, it is likely that there are multiple sources of stem and progenitor cells that converge into the MPC phenotype once they are exposed to the biochemical milieu of the traumatized muscle tissue, and this heterogeneity could account for differences between MPCs and the more homogeneous population of bone marrow derived MSCs.

Regardless of their origin, it appears that the muscle-derived MPCs may be a clinically useful population of autologous cells for regenerative medicine, particularly in orthopaedic applications [53]. The typical standard of care for musculoskeletal injuries is to debride to the wound margin until definitive closure is possible [31]. The results of this study indicate that the viable portions of debrided tissue, typically discarded as surgical waste, might instead be harvested to obtain MPCs, which can be used to augment the wound healing process. The MPCs exhibit the trophic functions that are characteristic of bone marrow derived MSCs, and they can be harvested without the need for an additional procedure, such as bone marrow aspiration, which may be painful and exposes patient to additional surgical risks. Although the immunoregulatory functions of MPCs may be less effective than MSCs on a per cell basis, the MPCs can be harvested from traumatized muscle tissue at concentrations that are orders of magnitude higher than MSCs from bone marrow (Table 1). Therefore, the overall immunoregulatory function of MPCs harvested per gram of muscle tissue should be at least as effective as the MSCs harvested per gram of bone marrow (Fig. 5B). Furthermore, the MPCs may be harvested at sufficient numbers for immediate clinical use without the need for *ex vivo* expansion. We are currently evaluating the use of autologous MPCs to promote regeneration of muscle, as well as other tissues that are typically damaged as a result of orthopaedic trauma, such as bone, nerve and blood vessels [54, 55]. MPCs may also be useful to manage graft *versus* host disease following transplant of orthopaedic tissues [56].

In this study, we have evaluated the traumatized muscle derived MPCs by quantitatively comparing their differentiation potential and trophic properties to bone marrow derived MSCs. The two cell types share many similarities, although the extent to which the MPC population is able to undergo osteogenic and chondrogenic differentiation appears to be limited. Both cell types appear to exhibit immunoregulatory and pro-angiogenic trophic functions, which are an important component of the regenerative benefit of MSCs. Therefore, the traumatized muscle derived MPCs appear to be an alternative source of autologous cells that are capable of performing the trophic functions that enhance tissue regeneration. The MPCs may have an advantage over bone marrow derived cells in cellular therapy applications that follow orthopaedic trauma since they may be harvested without performing an additional surgical procedure, they may not require *ex vivo* expansion, and they appear activated by the trauma to participate in the wound healing response. We are continuing to investigate the mechanisms that mediate these trophic abilities in the MPCs and their function during wound healing. Simultaneously, we are developing regenerative medicine and tissue engineering strategies that take advantage of these functions to promote tissue regeneration.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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